American Society for Microbiology | Journal of Clinical Microbiology®

CLINICAL VETERINARY MICROBIOLOGY

Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Is a Superior Diagnostic Tool for the Identification and Differentiation of Mycoplasmas Isolated from Animals

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ABSTRACT In veterinary diagnostic laboratories, identification of mycoplasmas is achieved by demanding, cost-intensive, and time-consuming methods that rely on antigenic or genetic identification. Since matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) seems to represent a promising alternative to the currently practiced cumbersome diagnostics, we assessed its applicability for the identification of almost all mycoplasma species isolated from vertebrate animals so far. For generating main spectrum profiles (MSPs), the type strains of 98 *Mycoplasma*, 11 *Acholeplasma*, and 5 *Ureaplasma* species and, in the case of 69 species, 1 to 7 clinical isolates were used. To complete the database, 3 to 7 representatives of 23 undescribed *Mycoplasma* species isolated from livestock, companion animals, and wildlife were also analyzed. A large in-house library containing 530 MSPs was generated, and the diversity of spectra within a species was assessed by constructing dendrograms based on a similarity matrix. All strains of a given species formed cohesive clusters clearly distinct from all other species. In addition, phylogenetically closely related species also clustered closely but were separated accurately, indicating that the established database was highly robust, reproducible, and reliable. Further validation of the in-house mycoplasma library using 335 independent clinical isolates of 32 mycoplasma species confirmed the robustness of the established database by achieving reliable species identification with log scores of $\geq$1.80. In summary, MALDI-TOF MS proved to be an excellent method for the identification and differentiation of animal mycoplasmas, combining convenience, ease, speed, precision, and low running costs. Furthermore, this method is a powerful and supportive tool for the taxonomic resolution of animal mycoplasmas.

KEYWORDS animal mycoplasmas, MALDI-TOF MS

Members of the class *Mollicutes* (termed here by their trivial name, mycoplasmas) are the smallest and simplest self-replicating organisms, distinguished from ordinary bacteria by their complete lack of a cell wall. Mycoplasmas are widespread in nature, with currently more than 130 validly described species detected or isolated in/from vertebrate animals. Most of these mycoplasmas are members of the genera *Mycoplasma*, *Ureaplasma*, and *Acholeplasma* (1). The taxonomy of genus *Mycoplasma* is a delicate and complex issue, and recently proposed changes to nomenclature (2) are currently under debate (3).

Several animal mycoplasma species are considered mere commensals, while others are recognized as opportunistic or primary pathogens causing mostly slowly progressive and chronic diseases (4, 5). Because of these differences in the clinical relevance of

Citation Spergser J, Hess C, Loncaric I, Ramírez AS. 2019. Matrix-assisted laser desorption ionization–time of flight mass spectrometry is a superior diagnostic tool for the identification and differentiation of mycoplasmas isolated from animals. J Clin Microbiol 57:e00316-19. https://doi.org/10.1128/JCM.00316-19.

Editor Brad Fenwick, University of Tennessee at Knoxville

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Received 26 February 2019
Returned for modification 30 March 2019
Accepted 14 June 2019
Accepted manuscript posted online 19 June 2019
Published 26 August 2019

different mycoplasma species, and indicated treatment, prevention, and control strategies, accurate species identification of animal mycoplasmas is highly desirable. Laboratory diagnosis of animal mycoplasmas is commonly achieved by conventional cultivation procedures or by PCR for the detection of uncultivable mycoplasmas (e.g., hemoplasmas), highly fastidious mycoplasma species, and mycoplasmas causing diseases of high veterinary importance (6). Cultivation procedures include examination of a limited number of biochemical properties, such as glucose fermentation and arginine or urea hydrolysis (7), followed by antigenic or molecular identification of mycoplasma isolates. Antigenic identification tests (8, 9), however, depend on specific antisera to each individual mycoplasma species not readily available in most diagnostic laboratories. In addition, specific antisera may vary in the capacity to identify mycoplasmas because of multiple cross-reactions and substantial serological heterogeneity of some mycoplasma species (10). Molecular identification of mycoplasma isolates is based on universal PCRs targeting the 16S rRNA gene, the 16S-23S intergenic spacer region (ISR), and the *rpoB* gene, followed by sequence or amplicon analyses (10–14). Nevertheless, both antigenic and molecular identification techniques are time-consuming, labor-intensive, and not always discriminating and require expertise which is rather restricted to specialized laboratories. Species-specific PCR systems for the identification of mycoplasma isolates are certainly more rapid and discriminatory, yet with more than 130 animal mycoplasma species currently recognized and with up to 20 mycoplasma species that can be present in a given animal host, molecular identification of mycoplasma isolates applying multiple individual PCRs is difficult to manage, precluding their employment in veterinary diagnostic laboratories.

In past years, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been used in diagnostic veterinary microbiology, enabling rapid and accurate species identification of a vast majority of microorganisms encountered in routine diagnostic laboratories (15–18). Yet the use of MALDI-TOF MS for the identification of mycoplasma isolates is hindered by a limited number of mycoplasma reference spectra in currently available databases. Although MALDI-TOF MS has been reported to be a useful tool for the identification of 10 human and 13 ruminant mycoplasmas (17, 19), as well as the rodent mycoplasma *Mycoplasma pulmonis* (20), a systematic and general assessment of this technique for the identification of almost all cultivable representatives of animal mycoplasmas is still missing. A well-characterized strain collection representing three genera (*Mycoplasma*, *Ureaplasma*, and *Acholeplasma*) and 13 phylogenetic clusters/groups of animal mycoplasmas (10) was therefore analyzed by MALDI-TOF MS. Overall, the potential of MALDI-TOF MS for the identification and differentiation of almost all animal mycoplasma species was efficiently assessed.

MATERIALS AND METHODS

Type strains and clinical isolates used for the construction of an animal mycoplasma reference database. For generating main spectrum profiles (MSPs), the type strains of 98 *Mycoplasma*, 11 *Acholeplasma*, and 5 *Ureaplasma* species as well as 1 to 7 epidemiologically unrelated clinical isolates of 69 species were used. To complete the in-house database, MSPs of 3 to 7 representatives of 23 undescribed *Mycoplasma* species were also included (all strains are listed in Table 1; further information on the strains' origin is given in Table S1 in the supplemental material). Strains were grown in modified SP4 (21, 22), Friis (23), Frey (24), or U4 (25) medium (differences in culture conditions are indicated in Table 1). Identities of type strains were confirmed by ISR sequencing as described previously (26). Clinical isolates were identified by 16S rRNA gene (27) and ISR sequencing. Representatives of putative new *Mycoplasma* species were selected based on their 16S rRNA gene, ISR, and/or partial *rpoB* gene (10) sequencing results (accession numbers listed in Table 1 and Table S2). All sequences were subjected to similarity search against the GenBank DNA database at https://www.ncbi.nlm.nih.gov/GenBank (28).

Protein extraction and generation of reference spectra. *Mycoplasma* and *Acholeplasma* cells were harvested in a class II biosafety cabinet from 1 ml of late-log-phase cultures by centrifugation at $20{,}000 \times g$ for 5 min. For 5 *Mycoplasma* and 2 *Acholeplasma* species (*M. dispar*, *M. flocculare*, *M. hyopneumoniae*, *M. microti*, *M. synoviae*, *Acholeplasma morum*, and *A. vituli*) a higher volume of 5 ml and for all *Ureaplasma* species a large volume of 100 ml were required for the generation of quality spectra. Supernatants were decanted, and pellets were washed twice with 200 μl of high-performance liquid chromatography (HPLC)-grade water (Sigma-Aldrich, Vienna, Austria). After centrifugation at $20{,}000 \times g$ for 5 min, pellets were subjected to formic acid-acetonitrile extraction as previously described (19), with

TABLE 1 Type strains and clinical isolates used in the current study to construct the in-house mycoplasma MSP database

Phylogenetic cluster/group	Taxon (closest relative, % similarity)[a]	Main host(s)	Type and field strain(s) (accession number for 16S rRNA gene, ISR, and/or *rpoB* gene sequence)	Culture conditions
Bovis-lipophilum cluster	*M. adleri*	Goat	G145^T, G43	SP4, 37°C
	M. agalactiae	Goat, sheep, wild ungulates	PG2^T, UBS343, Howd44, GA334, JT3, Murcia3, F4	
	M. bovigenitalium	Cattle	PG11^T, J6, CH2413, VBG11, U107, 85/2	
	M. bovis	Cattle	PG45^T, V208, M1, 85L, 381, 1345, 2493	
	M. californicum	Cattle	ST-6^T, 102, 136, 547, 249, 51, 66, 69	
	M. caviae	Guinea pig	G122^T, CC4	
	M. columbinasale	Pigeon	694^T, 126	
	M. columbinum	Pigeon	MMP 1^T, 1973, TE17a, TE22, 48868, 2327, 3026	
	M. felifaucium	Puma	PU^T	
	M. fermentans	Human	PG18^T, Viper1, 103GL, Weef, Waran	
	M. gallinarum	Chicken	PG16^T, B2-4/5, B4-2/3, B4-5/8, B5-3/3	
	M. hyopharyngis	Pig	H3-6B-F^T	
	M. iners	Chicken	PG30^T, 1236, B5-5/5, B10-5/9, 142, A1	
	M. lipofaciens	Chicken	R171^T	
	M. maculosum	Dog	PG15^T, 554, 2193I, 2107, 2931X, 231, 3162	
	M. meleagridis	Turkey	17529^T, P889, P911	
	M. mucosicanis	Dog	1642^T, 139, 1296, 1600, 2305, 2846, 3364	
	M. opalescens	Dog	MH 5408 D^T	
	M. phocirhinis	Pinniped	852^T	
	M. primatum	Grivet monkey	HRC292^T	
	M. simbae	Lion	LX^T	
	Mycoplasma sp. cattle (*M. primatum*, 97%)	Cattle	Zaradi2 (FM196534.1, FM196535.1, KX863552.1), Jux, Haberl, 310, 2282, 92K3	
	Mycoplasma sp. cormorant 2 (*M. maculosum*, 98%)	Great cormorant	19827 (KX786695.1, KX863544.1, KX863557.1), 19836, 20848, 20850	
	Mycoplasma sp. dromedary (*M. bovigenitalium*, 96%)	Dromedary	Jamara (MK554806, MK554828, MK561038), d6Ud, D3, D5	
	Mycoplasma sp. goose 1 (*M. caviae*, 94%)	Goose	2445/1 (KX786691.1, KX863540.1, KX863550.1), 2445/5, A45, 2659/9, 3976/3, 711/7	
	Mycoplasma sp. goose 2 (*M. spermatophilum*, 96%)	Goose	2445/3A (MK554807, MK554829, MK561039), B22, B457, 715/4	
	Mycoplasma sp. penguin 2 (*M. felifaucium*, 97%)	Humboldt penguin	1052 (KX786690.1, KX863539.1, KX863549.1), 1123, 2068K2, 2303, 2607, 2855 C	
Synoviae cluster	*M. alligatoris*	Alligator	A21JP2^T	SP4, 30°C
	M. anatis	Duck	1340^T, E1, E5, 649/3, 13478, E511	SP4, 37°C
	M. bovirhinis	Cattle	PG43^T, 2184, Bal6, 2781, 3014/6, 3432/14, Bal15, Bal72	
	M. buteonis	Buzzard	Bb/T2g^T, B6183, 48589A, 46852, 64895, 470057	
	M. canis	Dog, cattle	PG14^T, 2775, 2834, 856, 898X, 2244	
	M. ciconiae	Stork	ST 57^T	
	M. citelli	Richardson's ground squirrel	RG-2C^T	
	M. columborale	Pigeon	MMP 4^T, 118, 3556, 2628, 47977, 3895	
	M. corogypsi	Vulture	BV1^T	
	M. cricetuli	Chinese hamster	CH^T	
	M. cynos	Dog	H 831^T, 1334, 1046, 2297, 141, 283, 3619	
	M. edwardii	Dog	PG24^T, 2491, 1307, 2570, 2733, 4122	
	M. felis	Cat	CO^T, 1206, 2193, 734, 1370, 4234	

(Continued on next page)

TABLE 1 (Continued)

Phylogenetic cluster/group	Taxon (closest relative, % similarity)[a]	Main host(s)	Type and field strain(s) (accession number for 16S rRNA gene, ISR, and/or *rpoB* gene sequence)	Culture conditions
	M. gallinaceum	Chicken	DD^{T}, B1-8, B2-4/6, B12-4/1, 1866A, 483A	
	M. gallopavonis	Turkey, chicken	WR1^{T}, H74, C10	
	M. glycophilum	Chicken	486^{T}, ZG786, 3436, 3800	
	M. leonicaptivi	Lion	3L2^{T}	
	M. mustelae	Mink	MX9^{T}	
	M. oxoniensis	Chinese hamster	128^{T}	
	M. pullorum	Chicken	CKK^{T}, B5-2K1, A1p, MS125, 1603, 2041, 1888/1, 483B	
	Mycoplasma sp. cormorant 1 (*M. glycophilum*, 97%)	Great cormorant	19801, 19806 (KX786687.1, KX863536.1, KX863546.1), 19817, 19822, 20881, 20894, 20900	
	Mycoplasma sp. eagle 1 (*M. verecundum*, 98%)	Eagle	1449 (FM196532.1, FM196533.1, MK561040), SH20, SP48II, BRA290, AA7A, 4F, B6152	
	Mycoplasma sp. eagle 2 (*M. anatis*, 96%)	Eagle	AA1 (KX786693.1, KX863542.1, KX863553.1), HF6, HF8/C, M13mK1, AA7B, HF9	
	Mycoplasma sp. ground squirrel (*M. citelli*, 98%)	European ground squirrel	1579 (KX786692.1, KX863541.1, KX863551.1), 3682, 3795, 3799, 4620, 4627	
	Mycoplasma sp. hoopoe (*M. mustelae*, 95%)	Hoopoe	3166/6 (MK554805, MK554827, MK561037), 3166/7, 424, 365/4, T65, T93	
	Mycoplasma sp. ostrich 1 (*M. verecundum*, 95%)	Ostrich	2F1A (FM165076.2, FM196530.1, KX863555.1), 1FA, Ms03[y]	
	Mycoplasma sp. rail 1 (*M. columborale*, 97%)	Water rail	R1, R8, R12, R19A (KX786689.1, KX863538.1, KX863548.1), R24, R31	
	Mycoplasma sp. rail 2 (*M. pullorum*, 96%)	Water rail	R13 = EA139240 (KX786696.1, KX863545.1, KX863558.1), R19B, R21, R22, R35	
	Mycoplasma sp. sea lion (*M. edwardii*, 97%)	South American sea lion	Moneda (KX786688.1, KX863537.1, KX863547.1), Carmen2, 3948/11, Leo, O129	
	M. sturni	Starling	UCMF^{T}, 47832, 47837, 47860, 47950, 48488, ZD33618	
	M. synoviae	Chicken, turkey	WVU 1853^{T}, B3-4/8, Gla4, C13, 789, 1340	Frey, 37°C
	M. verecundum	Cattle	107^{T}	SP4, 37°C
Elephantis-equi-genitalium group	*M. elephantis*	Elephant	E42^{T}	SP4, 37°C
	M. equigenitalium	Horse	T37^{T}, Mister, 480I, 1023, 3057, 2488	
Agassizii-pulmonis-testudineum group	*M. agassizii*	Tortoise	PS6^{T}	SP4, 30°C
	M. pulmonis	Rat, mouse	PG34^{T}, 1854, 988, 2620, 2382o6, 3119	SP4, 37°C
	M. testudineum	Tortoise	BH29^{T}	SP4, 30°C
Hominis cluster	*M. alkalescens*	Cattle	PG51^{T}, 162, 2256, 897, 1203, M70	SP4, 37°C
	M. anseris	Goose	1219^{T}, 262/20, 1474/4, 3659/2, 3976/5, K769	
	M. arginini	Miscellaneous	G 230^{T}, MA3, 1264, 2352, 2493, 3450, 3817	
	M. arthritidis	Rat, mouse	PG6^{T}, 544, 1962	
	M. auris	Goat	UIA^{T}, Capote	
	M. canadense	Cattle	275C^{T}, W13K1, CH571, 1368, GI7, 2911	
	M. cloacale	Turkey, goose	383^{T}, 347, 1654/13, 2445/6, 201584/10, 4194	
	M. equirhinis	Horse	M432/72^{T}, 3358, 2889, 2736, 1028, 664	
	M. falconis	Falcon	H/T1^{T}, 743, J1, 3026, 48533B, T4C	
	M. gateae	Cat	CS^{T}, 1950, 1846, 86, 908, 2176, 756	
	M. gypis	Vulture	B1/T1^{T}, 2634G, M40m, Qu16, CG1, 654G	
	M. hyosynoviae	Pig	S16^{T}, L10, 3432/1, 986/1, 1792/3, 2788	
	M. neophronis	Vulture	G.A.^{T}	
	M. phocicerebrale	Pinniped	1049^{T}, Lunita, Stella, Carmen1	
	M. phocidae	Pinniped	105^{T}	
	M. spumans	Dog	PG13^{T}, 910X, 2461, 348, 2327, 2356	
	Mycoplasma sp. dog (*M. arginini*, 98%)	Dog	125 (MK554803, MK554825, MK561035), 228A, 415, 2268, 2978	

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TABLE 1 (Continued)

Phylogenetic cluster/group	Taxon (closest relative, % similarity)[a]	Main host(s)	Type and field strain(s) (accession number for 16S rRNA gene, ISR, and/or *rpoB* gene sequence)	Culture conditions
	Mycoplasma sp. ostrich 2 (*M. spumans*, 98%)	Ostrich	237IA (FM165077.2, FM196531.1, MF770747.1), 238A, 238B, VIA, VIB, S4, Ms01[d]	
	Mycoplasma sp. penguin 1 (*M. spumans*, 96%)	Humboldt penguin	A1802 (FM165075.1, FM196529.1, KX863560.1), 2068K1, 2670, 12B, 350, 2499	
	Mycoplasma sp. sparrowhawk (*M. neophronis*, 98%)	Eurasian sparrowhawk	48620KC (MK554802, MK554824, MK561034), 48625A, 46991B, 49978B	
	Mycoplasma sp. stone-curlew (*M. gypis*, 97%)	Eurasian stone-curlew	3145, 3176, 3177, 3221, 3222 (MK554804, MK554826), 3226	
	Mycoplasma sp. stork 1 (*M. gypis*, 97%)	White stork	St57K1 (KX786686.1, KX863535.1), St93K2, 1375B, Sp3	
	Mycoplasma sp. stork 2 (*M. spumans*, 97%)	White stork	48521 (MK554808, MK554830, MK561036), 1375 C, St93K3, 48339/1, 48861	
	M. subdolum	Horse	TB^{T}, 132, Marion, Nina, 2169, 2686	
Neurolyticum-hyopneumoniae cluster	*M. bovoculi*	Cattle	M165/69^{T}, 123, C987	Friis, 37°C
	M. collis	Rat, mouse	58B^{T}	
	M. conjunctivae	Goat, sheep, wild ungulates	HRC/581^{T}, 6999	Friis, 30°C
	M. dispar	Cattle	462/2^{T}, 497, 1698	Friis, 37°C
	M. flocculare	Pig	Ms42^{T}, S698, Cepa 11A2	
	M. hyopneumoniae	Pig	J^{T}, 7B, B31, B875, Ivv, 4284	
	M. hyorhinis	Pig, cell culture	PG42^{T}, 2730, 2926, 3051, 569, 2697, 3901, 2788	
	M. iguanae	Iguana	2327^{T}	
	M. lagogenitalium	Pika	12MS^{T}	
	M. molare	Dog	H 542^{T}	
	M. neurolyticum	Mouse	Sabin Type A^{T}, ZV1	
	M. ovipneumoniae	Sheep, goat, wild ungulates	Y98^{T}, GS19, 1839, 2167/3, 2605, 5310/4	
	Mycoplasma sp. badger (*M. lagogenitalium*, 98%)	Badger	480 (MK554801, MK561033), 1012, 1746, 2269	
Moatsi-mobile-sualvi group	*M. moatsii*	Monkey	MK 405^{T}	SP4, 37°C
	M. mobile	Fish	163K^{T}	SP4, 25°C
	M. sualvi	Pig	Mayfield B^{T}	SP4, 37°C
Mycoides cluster	*M. capricolum* subsp. *capricolum*	Goat	California kid^{T}, 363 C, Murcia2	SP4, 37°C
	M. capricolum subsp. *capripneumoniae*	Goat	F38^{T}	
	M. cottewii	Goat	VIS^{T}	
	M. feriruminatoris	Wild ungulates	G5847^{T}	
	M. leachii	Cattle	PG50^{T}	
	M. mycoides subsp. *capri*	Goat, wild ungulates	PG3^{T}, 280, 6 M, 956, BZ1, 824, 414, F13	
	M. mycoides subsp. *mycoides*	Cattle	PG1^{T}	
	M. putrefaciens	Goat	KS-1^{T}, 363 P	
	M. yeatsii	Goat	GIH^{T}	
Cavipharyngis-fastidiosum group	*M. cavipharyngis*	Guinea pig	117C^{T}	SP4, 37°C
	M. fastidiosum	Horse	4822^{T}	
Genitalium-pneumoniae cluster	*M. alvi*	Cattle	Ilsley^{T}	SP4, 37°C
	M. gallisepticum	Chicken	PG31^{T}, Vi85, L230, B77, Glatzl7, 3132l	
	M. imitans	Duck	4229^{T}, 92K1, 193K3	
	M. testudinis	Tortoise	1008^{T}	SP4, 30°C
	M. tullyi	Humboldt penguin	56A97^{T}, 2609	SP4, 37°C

(Continued on next page)

TABLE 1 (Continued)

Phylogenetic cluster/group	Taxon (closest relative, % similarity)[a]	Main host(s)	Type and field strain(s) (accession number for 16S rRNA gene, ISR, and/or *rpoB* gene sequence)	Culture conditions
Iowae-penetrans group	*M. iowae*	Turkey	695^{T}, 715665, 1358	SP4, 37°C
	M. microti	Vole	IL371^{T}	Friis, 37°C
	M. muris	Mouse	RIII-4^{T}	SP4, 37°C, aa[b]
Ureaplasma cluster	*U. canigenitalium*	Dog	D6P-C^{T}, H154, H36, U587, V69, U788	U4, 37°C
	U. cati	Cat	F2^{T}, K33, K567, K4	
	U. diversum	Cattle	A417^{T}, V33, U562, S680, S78, V005, U12	
	U. felinum	Cat	FT2-B^{T}, K12, K456, K89, K560, K1	
	U. gallorale	Chicken	D6-1^{T}	
Acholeplasma cluster	*A. axanthum*	Miscellaneous	S743^{T}, 2272	SP4, 33°C
	A. equifetale	Horse	C112^{T}	
	"*M. feliminutum*"[c]	Cat, cattle	Ben^{T}, V48, U102, V103, 388, 3385/7, 3480/22	
	A. granularum	Pig	BTS-39^{T}, 35	
	A. hippikon	Horse	C1^{T}	
	A. laidlawii	Miscellaneous	PG8^{T}, Bend, P2, S1120, 631, 39cl, 3654, 54	
	A. modicum	Bovine cell culture	PG49^{T}	
	A. morum	Fetal calf serum	72-043^{T}	
	A. oculi	Miscellaneous	19-L^{T}, 114/2, 478, 1293/1	
	A. parvum	Horse	H23M^{T}, F366	
	A. vituli	Fetal calf serum	FC 097-2^{T}	

[a]For genus *Mycoplasma*, the original names standing in nomenclature are used. Closest relatives are based on highest similarity values of partial 16S rRNA gene sequences.
[b]aa, anaerobic.
[c]Shown to be an *Acholeplasma* species (10).
[d]Previously described (37).

slight modifications. Briefly, pellets were directly mixed with equal volumes of 70% formic acid and acetronitrile (20 to 40 μl, depending on pellet size). Ethanol protein precipitation prior to formic acid-acetonitrile extraction was omitted, as earlier tests revealed inconsistent results in the quality of spectra/scores if performed. After centrifugation at $20{,}000 \times g$ for 2 min, 1 μl of protein extract was spotted onto a 96-target polished steel plate (Bruker Daltonics, Bremen, Germany) in eight replicates, air dried, and overlaid with 1 μl of α-cyano-4-hydroxycinnamic acid matrix solution (Bruker Daltonics).

Mass spectra were generated employing a Bruker microflex LT Biotyper (Bruker Daltonics), and data (from 240 laser shots in 40 shot steps, in linear, positive-ion mode with a 60-Hz nitrogen laser) were summarized automatically using the AutoXecute acquisition control software of Bruker FlexControl 3.4. A bacterial test standard (BTS; Bruker Daltonics) was used in each run for calibration purposes and as a quality control. For generating MSPs of type strains and clinical isolates, 24 individual mass spectra from eight different spots of protein extracts were performed. The quality of raw spectra was then carefully evaluated using FlexAnalysis 3.4 software, and spectra diverging from the cohort core, flatline spectra, and spectra displaying high background noise were deleted. After smoothing and baseline correction, a minimum of 20 spectra of high quality were selected for MSP creation using standard settings of the automated MSP creation functionality in MBT Compass Explorer 4.1. Resulting MSPs were consecutively added to the in-house mycoplasma project library, and after establishment, each MSP was compared to the database and spectral concordances expressed by log score values were documented (see below). Next, score-oriented dendrograms based on arbitrary distance matrix of generated MSPs were constructed using the correlation distance measure with the average linkage algorithm (MBT Compass Explorer 4.1). As distance levels are relative values normalized to a maximum of 1,000, distances were not compared between dendrograms.

Corresponding MSPs were also compared to those in the Bruker integrated taxonomy library, containing reference spectra of 10 *Mycoplasma* species (in total 17 strains, including type strains and clinical isolates of *M. alkalescens*, *M. arginini*, *M. bovirhinis*, *M. bovis*, *M. canis*, *M. gallinaceum*, *M. gallisepticum*, *M. hyorhinis*, *M. ovipneumoniae*, and *M. pullorum*) and 1 clinical isolate of *A. laidlawii*.

Validation of the established animal mycoplasma reference database. For validating the established MSP database, protein extracts of 335 clinical isolates (Table 2), previously identified by ISR sequencing, were spotted onto target plates and classified by matching MSPs of the in-house library. The degree of spectral concordance was expressed as a logarithmic identification score, which was interpreted according to the manufacturer's instructions, with score values of $\geq$2.00 considered to be acceptable for the identification at the species level and $\geq$1.70 acceptable for the identification at the genus level.

Testing of culture media, culture conditions, and mycoplasma concentrations. To identify influences of culture growth phases and mycoplasma concentrations for MALDI-TOF MS identification, three type strains of ruminant mycoplasmas were selected: *M. bovirhinis* PG43^T (glucose fermenting, Synoviae cluster), *M. alkalescens* PG51^T (arginine-hydrolyzing, Hominis cluster), and *M. bovis* PG45^T (non-glucose fermenting, non-arginine hydrolyzing, Bovis-lipophilum cluster). Each strain was grown in 50 ml of SP4 medium (initially inoculated with 10^5 CFU) at 37°C for 7 days. MALDI-TOF MS was daily performed on 1 ml of culture in triplicate, and CFU per milliliter were determined by colony counting after plating serial dilutions on SP4 agar. To determine effects of culture media on MALDI-TOF MS results, 12 type strains were grown in a culture medium (i.e., SP4, Friis, modified Hayflick [29], and Frey) differing from those indicated in Table 1, and log scores of corresponding reference spectra of the same strain were recorded. In addition, proteins of nonseeded culture media were extracted as described above and analyzed by MALDI-TOF MS, and resulting spectra were compared to those in the in-house mycoplasma library.

Data availability. Sequences were deposited in GenBank under accession numbers FM165075 to FM165077, FM196529 to FM196535, KX786686 to KX786693, KX786695, KX786696, KX863535 to KX863542, KX863544 to KX863553, KX863555, KX863557, KX863558, KX863560, MF770747, MK554801 to MK554808, MK554824 to MK554830, and MK561033 to MK561040 (Table 1; see also Table S1 in the supplemental material).

RESULTS

In total, 114 known species of genera *Mycoplasma*, *Acholeplasma*, and *Ureaplasma* and 23 undescribed *Mycoplasma* species were analyzed. Type strains and clinical isolates were confirmed and identified by ISR sequencing and comparison to sequences in GenBank, providing 100% identity to published sequences of type strains and, in the case of clinical isolates, sequence similarity values of >95 to 98%, depending on the phylogenetic position of the strain. Representatives of undescribed *Mycoplasma* species were selected based on their 16S rRNA, ISR, and partial *rpoB* gene sequences, with similarity values of $\leq$98.7%, 95%, and 90%, respectively (10), placing them as distinct species within the Bovis-lipophilum, Synoviae, Hominis, and Neurolyticum-hyopneumoniae clusters (Table 1).

From these type strains and clinical isolates, a large in-house mycoplasma library containing 530 MSPs was generated. By comparing each MSP to those in the in-house database, species and even subspecies (Mycoides cluster) represented by more than one strain were correctly identified, consistently producing log scores above 2.00. In

TABLE 2 Validation of the in-house mycoplasma library using 335 independent clinical isolates of 32 mycoplasma species frequently isolated from animals

Isolate identified by ISR sequencing (no.)	Hosts	No. of isolates with MALDI-TOF log scores (best correct match)		MALDI-TOF log scores of correctly identified isolates		Best incorrect match (maximum log score)
		≥1.80	≥2.00	Range	Mean	
M. agalactiae (10)	Goats	10	9	1.98–2.83	2.42	*M. bovis* (1.62)
M. alkalescens (10)	Cattle	10	10	2.25–2.71	2.55	*M. auris* (1.35)
M. anatis (10)	Ducks	10	10	2.33–2.97	2.70	*M. pullorum* (1.42)
M. anseris (9)	Geese	9	9	2.21–2.88	2.65	*M. cloacale* (1.49)
M. arginini (12)	Miscellaneous	12	9	1.86–2.79	2.24	*M. gateae* (1.54)
M. bovigenitalium (8)	Cattle	8	7	1.92–2.79	2.61	*M. mucosicanis* (1.29)
M. bovirhinis (12)	Cattle	12	10	1.89–2.88	2.62	*M. cynos* (1.48)
M. bovis (17)	Cattle	17	13	1.90–2.85	2.46	*M. agalactiae* (1.61)
M. canadense (8)	Cattle	8	8	2.32–2.91	2.74	*M. arginini* (1.53)
M. canis (14)	Dogs	14	14	2.13–2.79	2.52	*M. edwardii* (1.58)
M. cloacale (9)	Geese	9	8	1.88–2.73	2.34	*M. anseris* (1.60)
M. cynos (8)	Dogs	8	8	2.43–2.90	2.75	*M. canis* (1.52)
M. edwardii (11)	Dogs	11	10	1.92–2.89	2.38	*M. canis* (1.51)
M. felis (17)	Cats, horses	17	17	2.18–2.89	2.49	*M. glycophilum* (1.38)
M. gallinaceum (10)	Chicken	10	10	2.32–2.81	2.50	*M. gallopavonis* (1.34)
M. gallinarum (8)	Chicken	8	7	1.99–2.90	2.48	*M. columbinasale* (1.40)
M. gallisepticum (8)	Chicken, turkey	8	8	2.43–2.92	2.78	*M. imitans* (1.65)
M. gateae (11)	Cats	11	11	2.37–2.77	2.61	*M. arginini* (1.52)
M. glycophilum (6)	Chicken	6	4	1.92–2.88	2.55	*M. gallinaceum* (1.38)
M. hyopneumoniae (10)	Pigs	10	10	2.31–2.87	2.68	*M. flocculare* (1.63)
M. hyorhinis (17)	Pigs	17	12	1.80–2.82	2.17	*M. hyopneumoniae* (1.50)
M. hyosynoviae (11)	Pigs	11	11	2.37–2.93	2.67	*M. subdolum* (1.42)
M. iners (9)	Chicken	9	9	2.03–2.79	2.62	*M. gallinarum* (1.48)
M. maculosum (8)	Dogs	8	8	2.15–2.85	2.36	*M. adleri* (1.53)
M. mycoides subsp. *capri* (9)	Goats	9	9	2.14–2.78	2.39	*M. leachii* (1.64)
M. ovipneumoniae (12)	Sheep, goats	12	7	1.82–2.77	2.21	*M. hyorhinis* (1.49)
M. pullorum (7)	Chicken	7	7	2.22–2.81	2.72	*M. sturni* (1.52)
M. pulmonis (9)	Rats, mice	9	8	1.90–2.85	2.58	*M. agassizii* (1.43)
M. spumans (16)	Dogs	16	13	1.83–2.92	2.36	*M. neophronis* (1.52)
M. synoviae (15)	Chicken, turkey	15	12	1.89–2.86	2.68	*M. verecundum* (1.38)
A. laidlawii (9)	Miscellaneous	9	9	2.11–2.82	2.48	*A. oculi* (1.38)
A. oculi (5)	Miscellaneous	5	3	1.98–2.92	2.43	*A. morum* (1.42)
Total (335)		335	300	1.80–2.97	2.52	

addition, comparable results were obtained for almost all taxa represented only by their type strain, all exhibiting unique MSPs, except *M. cottewii* and *M. yeatsii*, both producing log scores above 2.00 for each other. For all other species, best incorrect matches were considerably low, never exceeding log scores of 1.70. Species differentiation and diversity of spectra within a species were also assessed by constructing dendrograms based on a similarity matrix deduced from comparison of MSPs within a phylogenetic cluster. All strains of a species formed a cluster clearly distinct from other species (Fig. 1). Even highly related species of the Genitalium-pneumoniae cluster (Fig. 1D) and (sub)species of the Mycoides cluster (Fig. 1F) were unequivocally separated. However, within the Mycoides cluster, distance levels separating *M. cottewii* and *M. yeatsii* and subspecies of *M. mycoides* were highly similar (approximately 200) and far below the lowest distance level for species separation at 500, confirming the inability of MALDI-TOF MS to distinguish *M. cottewii* and *M. yeatsii*. Furthermore, phylogenetically highly related species such as *M. canis* and *M. edwardii* (Fig. 1A), *M. bovis* and *M. agalactiae* (Fig. 1B), *M. spumans* and *M. neophronis* (Fig. 1C), and *M. flocculare* and *M. hyopneumoniae* (Fig. 1E) clustered closely and the overall topology of score-oriented dendrograms was largely comparable to that of 16S rRNA phylogeny.

When MSPs of 10 *Mycoplasma* species and *A. laidlawii* were compared to those corresponding in the commercial Bruker database, it was found that the best matches

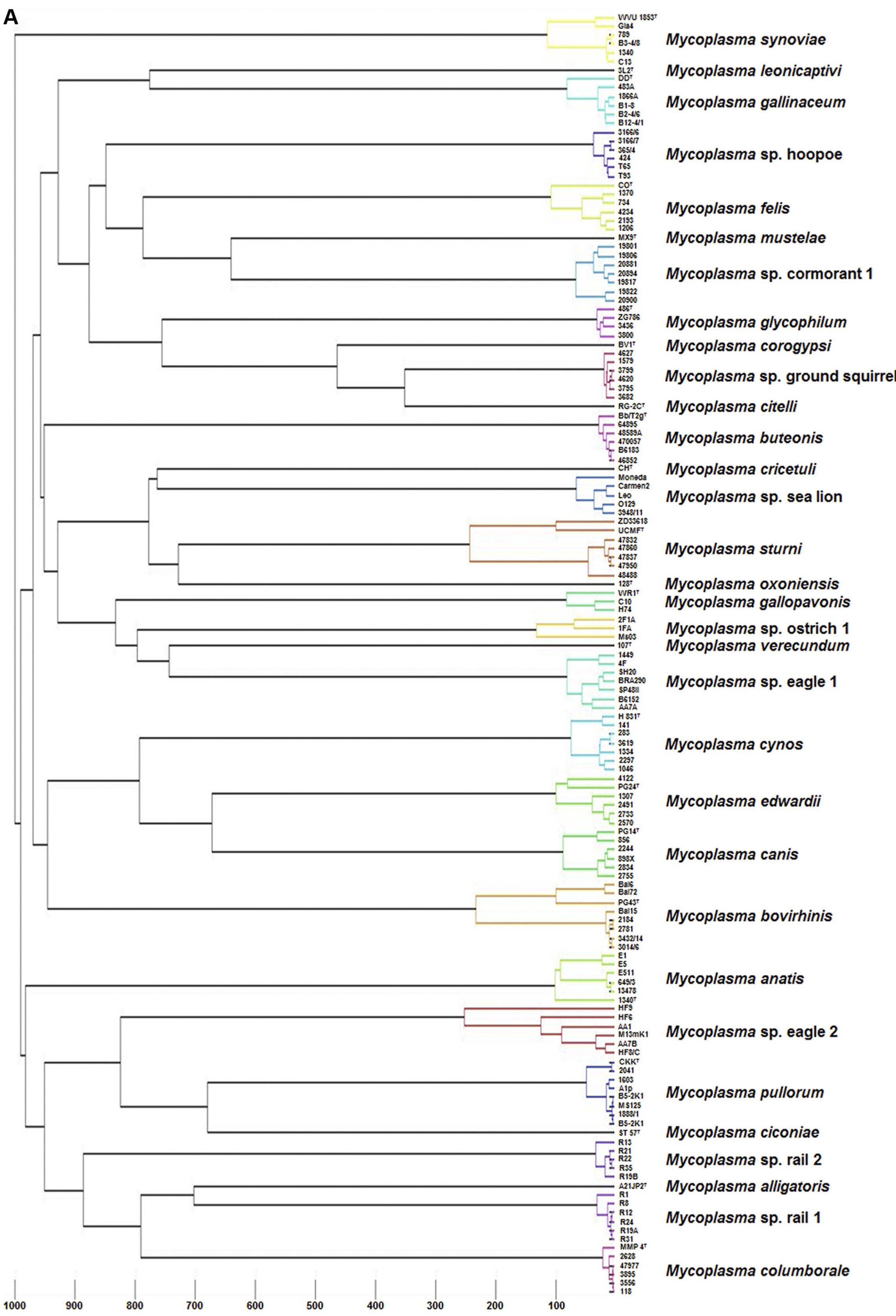

FIG 1 Dendrograms derived from similarity matrices based on MSPs from animal mycoplasmas of the phylogenetic Synoviae cluster (A), Bovis-lipophilum cluster (B), Hominis cluster (C), Genitalium-pneumoniae cluster (D), Neurolyticum-hyopneumoniae cluster (E), Mycoides

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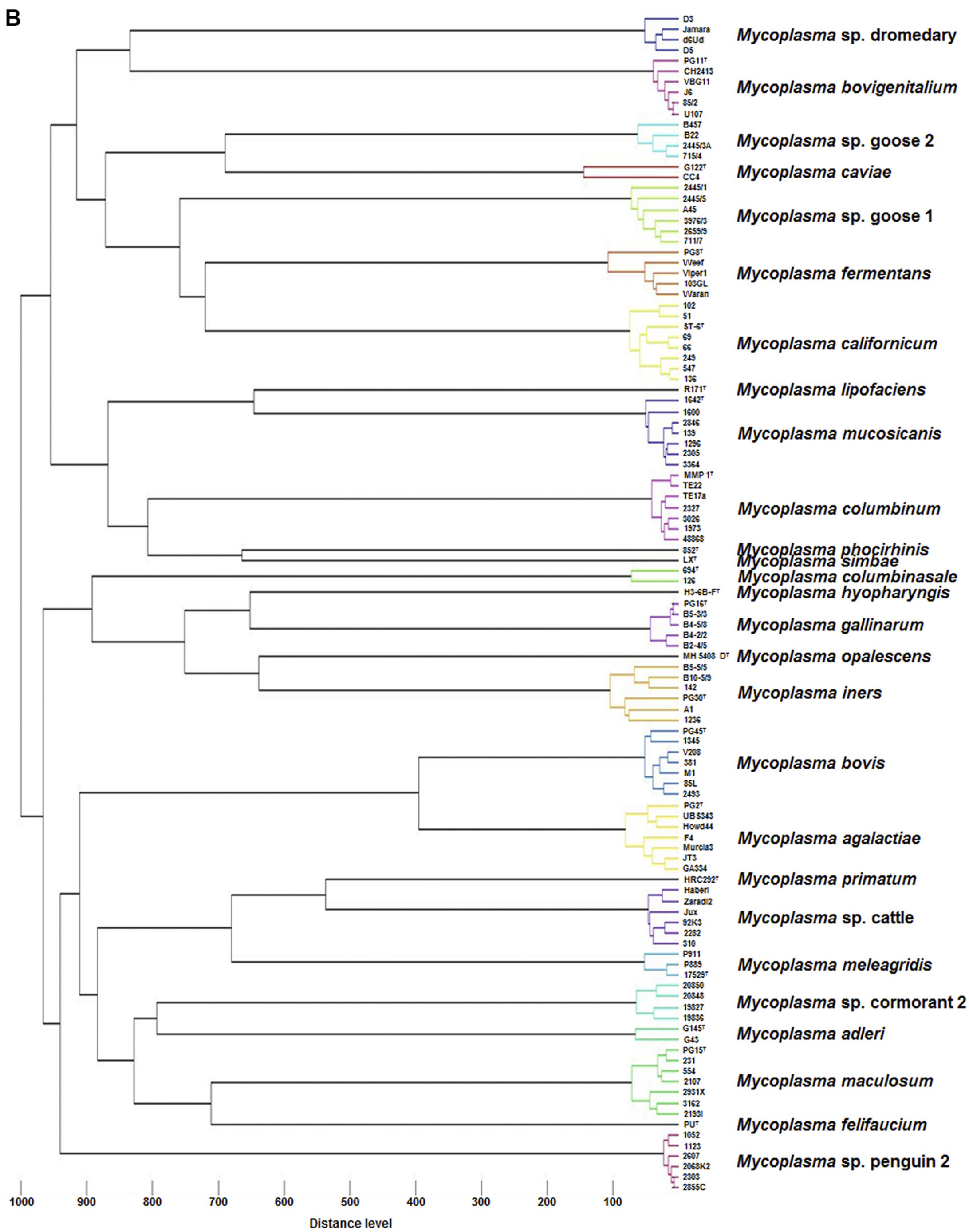

FIG 1 (Continued)

were from <1.70 (30% of strains tested) to between 1.70 and 2.00 (32% genus identification success) to >2.00 (38% species identification success).

The in-house mycoplasma library was further validated by testing a panel of 335 independent clinical isolates, identified by ISR sequencing and belonging to 32 species

FIG 1 Legend (Continued)

cluster (F), Ureaplasma cluster (G), and Acholeplasma cluster (H). Dendrograms illustrate species differentiation, cluster formation, diversity within species, and grouping of phylogenetically highly related species. Note that (i) distance levels separating *M. cottewii* and *M. yeatsii* and subspecies of *M. mycoides* are highly similar (approximately 200) and far below the lowest distance level for species separation (500) within the Mycoides cluster (F), and (ii) as distance levels are relative values normalized to a maximum of 1,000, distances are not comparable between dendrograms.

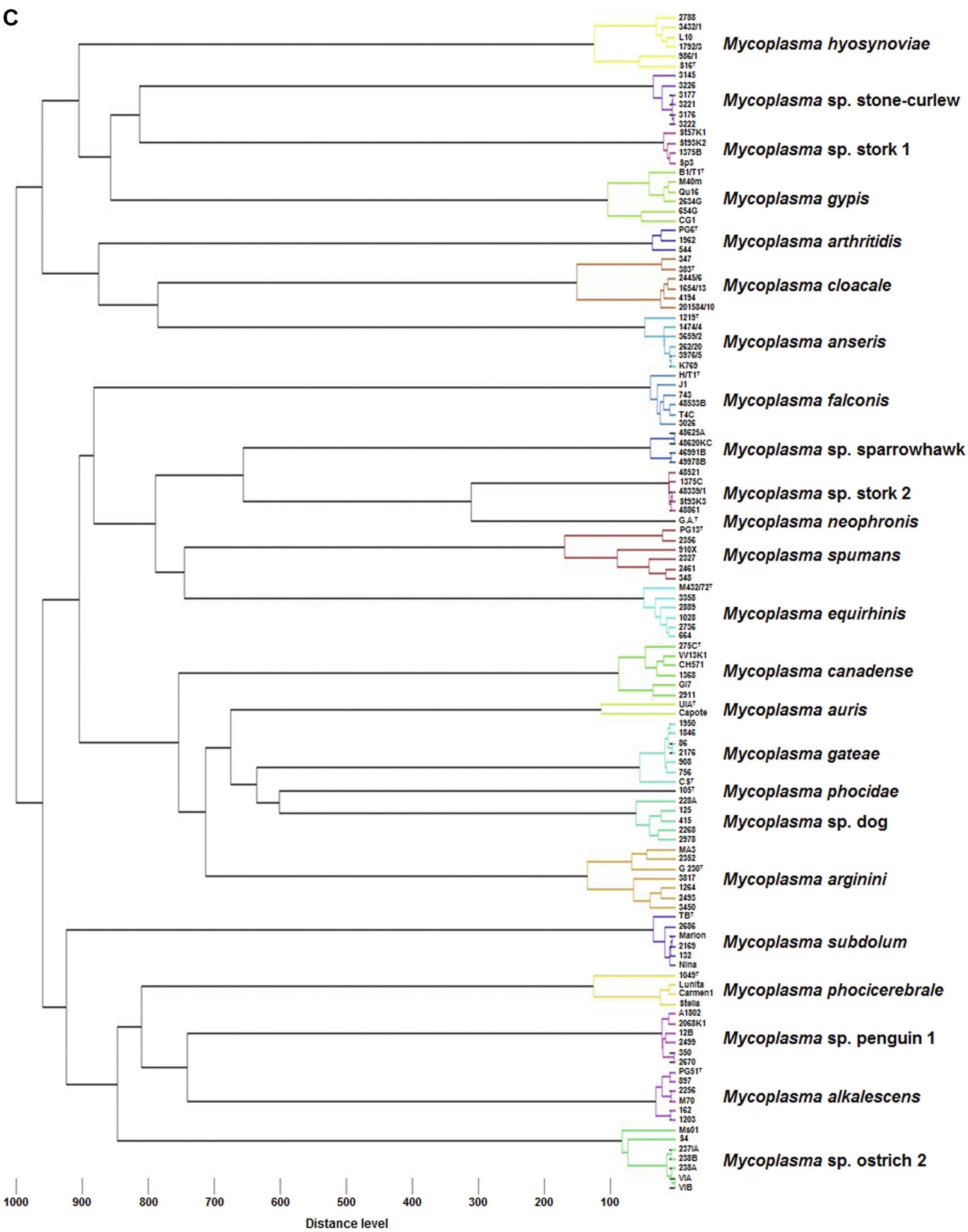

FIG 1 (Continued)

that are frequently isolated from clinical specimens in veterinary diagnostic laboratories. Best correct log scores for all 335 isolates exceeded 1.80 (100%), with 300 isolates (89.6%) producing best correct log scores above 2.00. Considerably low log scores of the best incorrect match (all <1.70) were obtained in most cases (Table 2).

To identify the best conditions for MALDI-TOF MS analysis, three type strains representing three phylogenetic groups and metabolic types were used for comparing mycoplasma concentrations and culture durations. Species identification (log scores ≥ 1.80) for all three strains was observed with all tested culture conditions when 10^6

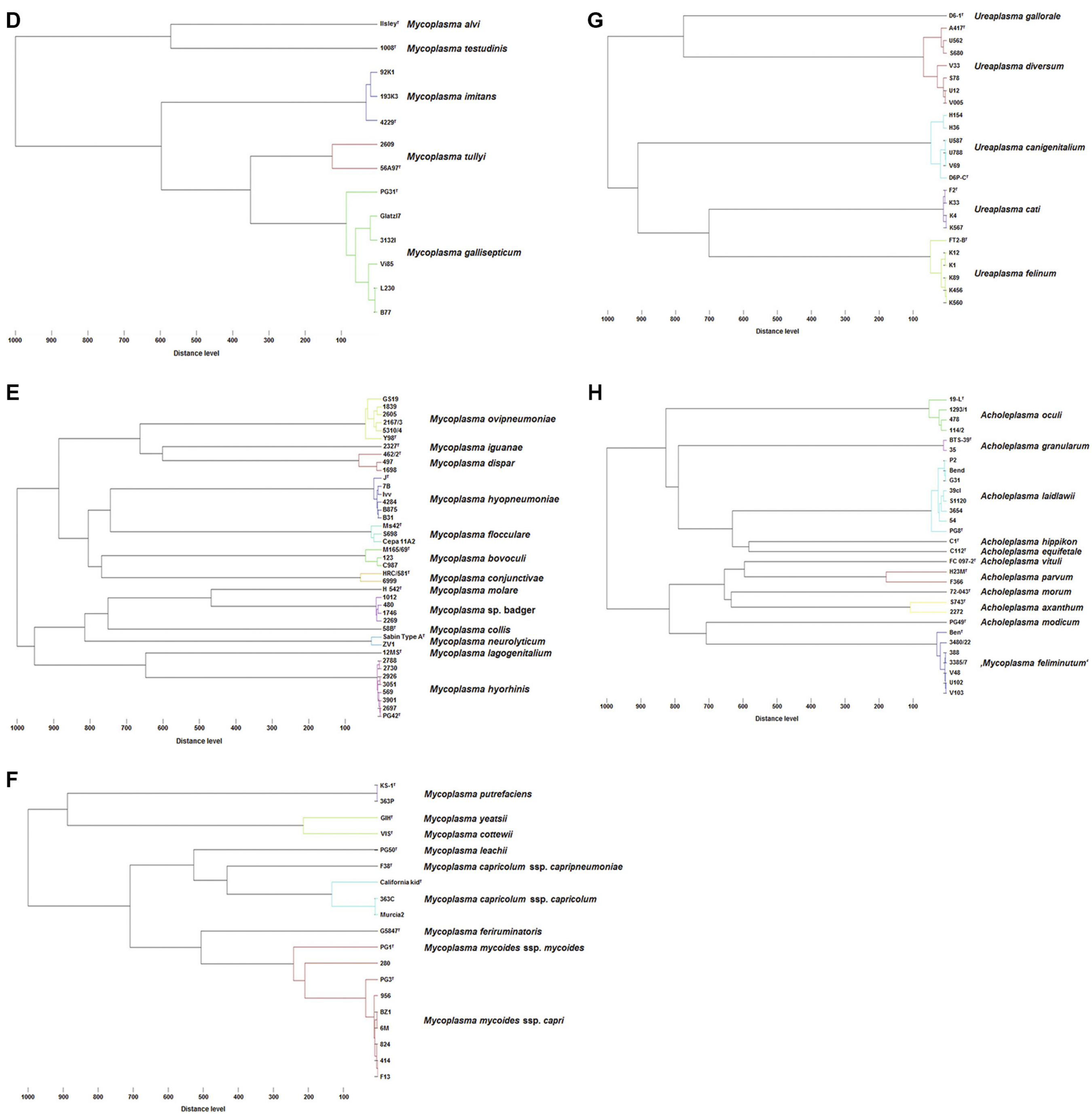

FIG 1 (Continued)

CFU/ml were obtained. Best identification scores were achieved when MALDI-TOF MS analysis was performed with cultures in the exponential phase of growth containing 10^7 to 10^8 CFU/ml depending on the strain tested, and scores remained above 1.80 in the stationary phase until day 7 of cultivation (Table 3).

For type strains grown in culture media differing from those used for generating reference spectra (Table 1), some degree of variation in the mass spectra was evident, but in general, the peak patterns derived by MALDI-TOF MS were stable and all type strains were accurately identified, with log scores above 2.00.

No spectral concordances were obtained by testing unseeded culture media.

TABLE 3 Culture growth phase- and mycoplasma concentration-dependent MALDI-TOF MS identification of *M. alkalescens* PG51^T, *M. bovirhinis* PG43^T, and *M. bovis* PG45^T

		Value for[a]:		
Day	Parameter	*M. alkalescens* PG51^T	*M. bovirhinis* PG43^T	*M. bovis* PG45^T
1	Mean CFU/ml Mean log score	2.1×10^4 **<1.70**	1.3×10^4 **<1.70**	3.7×10^4 **<1.70**
2	Mean CFU/ml Mean log score	3.9×10^5 **<1.80**	1.2×10^5 **<1.80**	8.7×10^6 2.13
3	Mean CFU/ml Mean log score	1.1×10^7 2.22	2.8×10^6 1.92	5.8×10^8 2.83
4	Mean CFU/ml Mean log score	8.8×10^7 2.54	5.3×10^7 2.78	7.8×10^8 2.51
5	Mean CFU/ml Mean log score	1.2×10^8 2.31	1.3×10^8 2.24	8.1×10^8 2.35
6	Mean CFU/ml Mean log score	3.9×10^7 1.88	6.3×10^7 1.81	9.4×10^7 2.11
7	Mean CFU/ml Mean log score	7.3×10^6 1.82	3.8×10^6 1.81	1.2×10^7 1.82

[a]Log scores of <1.80 are in bold.

DISCUSSION

Laboratory diagnosis of animal mycoplasmas is still widely based on cultural isolation of these bacteria from mucous membranes, secretions, or tissues followed by serological or molecular identification. However, these identification approaches are challenging, time-consuming, labor-intensive, and expensive, often precluding their employment in a routine diagnostic laboratory setting. With these limitations in mind, we assessed the applicability of MALDI-TOF MS for the identification of almost all known cultivable mycoplasmas isolated from vertebrate animals so far. Since only a limited number of animal mycoplasmas were represented in the latest Bruker MSP database, we first attempted to establish a large in-house MSP library representing three genera and 13 phylogenetic groups within the class *Mollicutes*, comprising 114 known and 23 undescribed taxa of animal mycoplasmas. Several previous reports have emphasized the importance of supplementing MSP databases of a given species with appropriate reference spectra of multiple strains in order to cover the natural diversity of the species and to improve identification success (18, 30, 31). Consequently, besides the type strains of 69 known animal mycoplasmas, 1 to 7 epidemiologically unrelated clinical isolates and 3 to 7 representatives of undescribed *Mycoplasma* species were included. Evaluation of this large mycoplasma database revealed that all organisms of a given species represented by more than one strain were correctly identified, indicating that the established mycoplasma database was highly robust, obtaining reproducible and reliable results. Forty-five taxa, mostly originating from exotic animals or wildlife, were represented only by their type strains, precluding full evaluation of the robustness of the MSPs generated. As log scores of the nearest matches were low (<1.60), the MSPs, however, appeared to be unique. Similar results were obtained in a former study including 29 type and reference strains of ruminant and human mycoplasmas in which a high discrimination at the (sub)species level was observed (19). In the present study, MALDI-TOF MS only failed in discriminating between two members of the Mycoides cluster, namely, *M. cottewii* and *M. yeatsii* (both represented only by their type strains). This result was surprising, as MALDI-TOF MS was shown to be able to differentiate all remaining members of the clusters even at the subspecies level (Fig. 1F). However, a previous phylogenetic study on *Mycoplasmataceae* discloses that *M. cottewii* and *M. yeatsii* exhibit the highest similarity values of 16S rRNA, ISR, and partial *rpoB* gene sequences (99.7%, 99.7%, and 97.7%, respectively) ever observed between two *Mycoplasma* species (10), indicating that further investigations including whole-genome comparisons are required in order to confirm that *M. cottewii* and *M. yeatsii* are truly two different *Mycoplasma* species.

When MSPs of 11 animal mycoplasma species were compared to corresponding MSPs in the Bruker database, identification according to Bruker's recommendation was limited to the genus level for 32% of strains tested. In contrast, 38% of strains were correctly identified, whereas 30% remained undiagnosed. Previous reports demonstrated that identification rates of MALDI-TOF MS may be influenced by the employment of different culture media, culture conditions (including incubation time), and storage conditions (31, 32), a low number of reference spectra in the database (18, 30), and even if newly generated MSPs of reference strains were used instead of those available in commercial databases (16). As culture media and conditions had no significant influence on identification in the current study, misidentification using the commercial database might be explained only by the two last influencing factors stated.

Further validation of the in-house mycoplasma library using 335 independent clinical isolates confirmed the robustness of the established in-house database with log scores of ≥1.80 to be reliable for species identification (Table 2). Validation, however, was limited to the 32 most frequently isolated animal mycoplasmas; thus, a notable number of animal mycoplasma species included in the established database remain unvalidated.

Although Bruker advocates log scores of ≥1.70 and ≥2.00 for genus- and species-level identification, our results suggest a reduction of the species-level identification threshold for mycoplasma identification. Such species-level threshold adaptations have been previously proposed for mycoplasmas and other microbial taxa (17, 19, 32, 33), allowing 100% species identification rates without any misidentification. On the other hand, isolates producing log scores at the genus-level identification range may represent atypical strains of a species; thus, including MSPs of those strains in the database in order to cover the diversity of mass spectral patterns of a given species is recommended. High intraspecific variability of mass spectral fingerprints may also point to the mycoplasma subtyping capability of MALDI-TOF MS as previously shown for *M. pneumoniae*, *M. bovis*, and *M. agalactiae* (19, 34, 35).

A protein extraction protocol, slightly modified during the current study, was applied to obtain high-quality spectra and to increase rates of identification. By omitting ethanol precipitation from a series of centrifugation and resuspension steps, sample preparation was further optimized to be compatible with the laboratory diagnostic workflow. For the generation of MSPs and for clinical identification, 1- to 5-ml cultures in the exponential phase of growth were appropriate to obtain qualified and interpretable spectra for all *Mycoplasma* and *Acholeplasma* species. In contrast, identification of *Ureaplasma* species required culture volumes of 100 ml, precluding the use of MALDI-TOF MS for the identification of animal ureaplasmas in routine diagnostic settings, which has already been shown for human *Ureaplasma* species (19).

The taxonomy of *Mollicutes* species, i.e., the establishment and definition of a species within this taxonomic category, still relies on polyphasic characterization of phenotypic and genetic features (36). Since currently used phenotypic tests are limited, labor-intensive, and not always discriminating, the development and evaluation of further phenotypic test approaches for the identification and characterization of cultivable mycoplasma species are required (10). Our results strongly suggest that MALDI-TOF MS may contribute to the taxonomy of *Mollicutes* and the description of new mycoplasma species obviously occurring in a wide range of different animals. In the current study, discrimination and ambiguous identification of yet-undescribed *Mycoplasma* species using MALDI-TOF MS has been demonstrated, since all strains of a single new taxon, genetically defined by 16S rRNA gene, ISR and partial *rpoB* gene sequencing (Table S2), were recognized as being members of the same species. Thus, MALDI-TOF MS may be used as a screening method for new mycoplasma species inhabiting animals, which may subsequently be characterized in order to extend our knowledge on their veterinary importance, epidemiology, diversity, and evolutionary biology.

Conclusion. In the present study, a large in-house spectral library of animal mycoplasmas, including undescribed *Mycoplasma* species, has been developed that proved to be reliable for the identification of clinical isolates. In conclusion, MALDI-TOF MS is an excellent method for the identification and differentiation of mycoplasmas isolated from animals and a powerful and supportive tool for the taxonomic resolution of animal mycoplasmas.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00316-19.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

We are grateful to Martina Zimmermann, Munkhtsetseg Kargl, and Michael Steinbrecher for their excellent technical assistance.

This study was supported by internal funding of the Institute of Microbiology, University of Veterinary Medicine Vienna.

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